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The truffle genus *Pachyphloeus* in the U.S. and Mexico: phylogenetic analysis and a new species

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Abstract—A molecular analysis of LSU and ITS portions of rDNA from *Pachyphloeus* ascocarp collections in the United States and Mexico gives strong bootstrap support for four clades within the genus. Two clades include collections from Iowa and Mexico. From one of these, a new species of *Pachyphloeus,* is described from oak woodlands. *Pachyphloeus marroninus* is distinguished from other species of *Pachyphloeus* by the combination of a reddish brown peridium with low, polygonal warts, a solid, white gleba, narrowly clavate asci, and spore spines that are coarse with tips free from the perisporium at maturity. Molecular analyses support the close relationship of this species from Iowa and Mexico, but the variations in sequences may indicate a cryptic species complex.

Key words— ascomycete, taxonomy, hypogeous fungi

Introduction

The genus *Pachyphloeus* was described by Tulasne & Tulasne (1844) based on *P. melanoxanthus* (Tul. & C. Tul. ex Berk.) Tul. & C. Tul. with the following characteristics: 1.) a thick peridium with an outer layer of pigmented cells; 2.) a thick margined orifice stuffed with hyphae, where the interstitial veins come to the surface in a depression on the ascoma; 3.) a prominent basal mycelial tuft; 4.) sterile veins that initially differ in color from the fertile tissue, but later resemble the fertile tissue in color; 5.) and asci with eight globose spores ornamented with spines and irregularly arranged in the ascus. The name *Pachyphloeus* was derived from a combination of Greek words for "thick" and "cortex" (Pegler et al. 1993), a tribute to the ample peridium.

To date *Pachyphloeus* contains 12 described species and one variety: *P. austro-oregonensis* J.L. Frank & Trappe (Frank et al. 2006), *P. carneus* Harkn. (1899), *P. citrinus* Berk. & Broome (1846), *P. conglomeratus* Berk. & Broome (1846), *P. lateritius* Fogel & States (2002), *P. ligericus* Tul. & C. Tul. (1851), *P. macrosporus* Calonge (Calonge et al. 2002), *P. melanoxanthus*, *P. melanoxanthus* var. *xanthocarnosus* Soehner (1936), *P. prieguensis* Mor.-Arr. et al. (Moreno-Arroyo et al. 1996), *P. saccardoi* Mattir. (1903), *P. thysellii* W. Colgan & Trappe (2004), and *P. virescens* Gilkey (1939).

A survey of hypogeous fungi in Iowa from 1997–2000 revealed several undescribed truffle species (Healy 2002, 2003, and unpublished data). Forays for hypogeous fungi in various locations in Mexico from before 1970 to 2008 produced many new records and undescribed species (Trappe & Guzmán 1971, Hosford & Trappe 1980, Cázares et al. 1992, and unpublished data). Morphological and molecular comparisons among similar collections of *Pachyphloeus* from both Iowa and Mexico indicate that some of these undescribed species are closely related. The first such species is described here.

Materials and methods

Data on color and morphology of fresh ascomata (Weber et al. 1997) were recorded, and the specimens photographed and air dried or prepared for microscopy. Colors matched to plates in Ridgway (1912) in quotation marks are followed by the INCC-NBS equivalent (Kelly & Judd 1955) in parentheses. Spores from air-dried specimens were measured in water under 100x. Fifty mature spores from each collection were measured. The holotype and some paratypes of *P. marroninus* sp. nov. were deposited in the Ada Hayden Herbarium at Iowa State University (ISC). Isotypes and other paratypes were deposited at the Oregon State University herbarium (OSC), Instituto Tecnologico de Ciudad Victoria (ITCV), Universidad Autónoma de Nuevo Leon (UNL) and Universidad Autónoma de Tlaxcala (TLXM). Specimens prepared for microscopy were fixed in FAA, embedded in paraffin, sectioned at 10 µm, stained in hematoxylin, and photographed with an Olympus camera. For scanning electron microscopy, paraffin embedded material was sectioned to 50 µm, deparaffinized in xylene, substituted with absolute ethanol, critical point dried in a DCP-1 Denton Critical Point Drying Apparatus (Denton Vacuum Inc., Cherry Hill, NJ), mounted on aluminum stubs with double-sided tape, silver painted around the specimen edges, sputter coated for 120 sec with Au/Pd, and visualized with 10 kV in a JEOL 5800LV SEM. Images were digitally captured. Imaging was done at the Bessey Microscopy Facility at Iowa State University.

Other species of *Pachyphloeus* from the collections of Healy, and J. Trappe were examined microscopically and many of these were included in the molecular analyses. These latter specimens are listed with their collection information and GenBank accession numbers in Table 1.

For molecular analysis, DNA was extracted from previously unexposed portions of the gleba of mature, air-dried ascocarps. Total DNA was extracted with 24:1 chloroform

Table 1 Collection data and GenBank numbers for sequences used in this study.*

Taxon	Voucher ID	Geographic origin, date, collector, herbarium of deposit, canopy spp.	GenBank #
Pachyphloeus cf. *carneus*	RH800	USA: IA, 9-8-00 Healy (ISC), *Quercus alba*	EU543199
P. cf. *carneus*	JT11019	MEX: NL, 10-22-88 Cázares, Trappe, Arnulfo (OSC), *Quercus,* mixed forest	EU543200
P. cf. *carneus*	RH20	USA: IA, 8-31-96 Healy (ISC), *Quercus macrocarpa*	EU543201
P. cf. *carneus*	RH725	USA: IA, 7-27-00 Healy (ISC), *Quercus macrocarpa*	EU543202
P. cf. *carneus*	RH525	USA: IA, 8-8-99 Healy (ISC), under *Quercus macrocarpa*	EU543203
P. cf. *carneus*	RH12	USA: IA, 7-9-07 Healy (ISC), *Quercus alba*	EU543204
P. cf. *carneus*	RH572	USA: IA, 9-9-99 Healy (ISC), *Tilia americana* and *Carya ovata*	EU543205
P. cf. *carneus*	RH756	USA: IA, 8-9-00 Healy (ISC), *Quercus macrocarpa*	EU543206
P. cf. *carneus*	RH518	USA: IA 8-6-99 Healy (ISC), *Quercus alba* and *Carya ovata*	EU543207
P. aarneus	Saylor2026	USA: CA 6-30-84 Saylor (OSC), *Lithocarpus densiflora*	AY500544 [1]
P. aarneus	JT12818	USA: CA 5-5-78 Heblack (OSC), *Quercus agrifolia*	EU543208
P. citrinus	JRWL2497	ITA: Cuneo, 12-9-00 (OSC), Mixed conifer and broadleaved trees	EU543196
P. marroninus	RH299	USA: IA 10-23-98 Healy (ISC, OSC), *Quercus rubra*	EU427549
P. marroninus	RH286	USA: IA 10-2-98 Healy (ISC, OSC), *Quercus alba* and *Q. macrocarpa*	EU427550
P. marroninus	García 3757	MEX: Nuevo Leon 9-14-83 Garcia (UNL), *Quercus polymorpha*	EU427551
P. marroninus	JT32454	MEX: Tlaxcala 9-20-07 Cázares (TLXM), *Quercus rugosa*, and *Q. crassifolia*	EU543209
P. cf. *melanoxanthus*	RH735	USA: IA, 7-28-00 Healy (ISC), *Quercus alba* and *Tilia americana*	EU543193
P. cf. *melanoxanthus*	RH195	USA: IA, 8-4-98 Healy & Gardner (ISC), *Quercus alba*	DQ191674[2]
P. melanoxanthus	MM1860	ITA: Venezia, Jan. 1999 Macchioni (OSC), *Ostrya* and *Quercus*	EU543194
P. virescens	RH279	USA: IA, 7-27-00 Healy (ISC), *Quercus macrocarpa*	EU543198
P. thysellii	JT13182	USA: WA, 8-19-93 Colgan (OSC), *Pseudotsuga menziesii*	EU543197
Pachyella clypeata		USA: IA, 9-18-05 Healy (ISC), riparian deciduous woods	EU543195
Peziza infossa		USA: CA	DQ974817[3]
Peziza cf. *badio-confusa*	RH7	USA: IA, 5-28-06 Healy (ISC), oak-hickory woods	EU571229

* 1. Hansen et al. 2005 as *P. citrinus*; 2. Tedersoo et al. 2006; 3. Smith et al. 2007.
All other sequences originate from this study.

: isoamyl alcohol. Both the internal transcribed spacer region (ITS1, 5.8S, and ITS2) and part of the ribosomal large subunit (LSU) locus were amplified using universal fungal primer set ITS5 - LR5 (Bertini et al. 1999, Vilgalys & Hester 1990). The PCR protocol began with an initial denaturation at 94 °C (3 min), followed by 35 cycles at

94 °C (2 min), 50 °C annealing (45 sec), and a 72 °C extension (1.5 min), with a final extension at 72 °C (7 min). Each 25 µl PCR reaction consisted of 4 µl dNTPs (1.25 µM), 2.5 µl PCR buffer, 1 µl BSA, 1.25 µ primer 1 (10 µM), 1.25 µl primer 2 (10 µM), 0.15 µl *Taq* DNA polymerase (1 µM/µL), 4.8 µl water, and 10 µl DNA extract (~10 ng / µl). Two µl of each PCR product was loaded into a 1.0% agarose gel buffered with TAE buffer and stained with 2 µl SYBR safe (Invitrogen, Carlsbad, CA) per 80 ml gel. Gel electrophoresis products were viewed on a GelDoc XR imager (Bio-Rad Laboratories, Inc., Hercules, CA). Qiagen Quick-Clean columns were used to clean PCR products prior to sequencing. Sanger bidirectional sequencing was performed using Big Dye chemistry version 3.1 (Applied Biosystems, Foster City, CA) with ITS5 (forward) and LR5 (reverse). DNA sequences were determined on an ABI3700 (Applied Biosystems, Foster City, CA).

DNA sequences were manually edited by use of Sequencher 4.0 (Gene Codes, Ann Arbor, MI), forward and reverse sequences were assembled, and ambiguous regions at the ends were trimmed. Both ITS and LSU sequences were queried against the NCBI public database GenBank with the BLASTN algorithm to compare with other sequences and to verify that sequences were of the target group. DNA sequences were aligned manually using MacClade 4.0 (Maddison & Maddison 2002). Ambiguously aligned regions were excluded from the alignment. Phylogenetic analysis was conducted on both the ITS and LSU alignments individually and combined, with unweighted parsimony heuristic criteria using PAUP 4.0b10 with 1000 random addition sequences and 5000 bootstrap replicates (Swofford 2001). Two independent maximum likelihood analyses based on a general-time-reversible 6-parameter model of evolution were run using the software program GARLI and included 100 bootstrap replications (Zwickl 2006). Outgroups in these analyses were chosen on the basis of preliminary data on phylogenetic relationships across the genus *Pachyphloeus* (Læssøe & Hansen 2007). The ITS and LSU sequences produced in this study were deposited in GenBank under the accession numbers EU427549–EU427551, EU543193–EU543209 and EU571229.

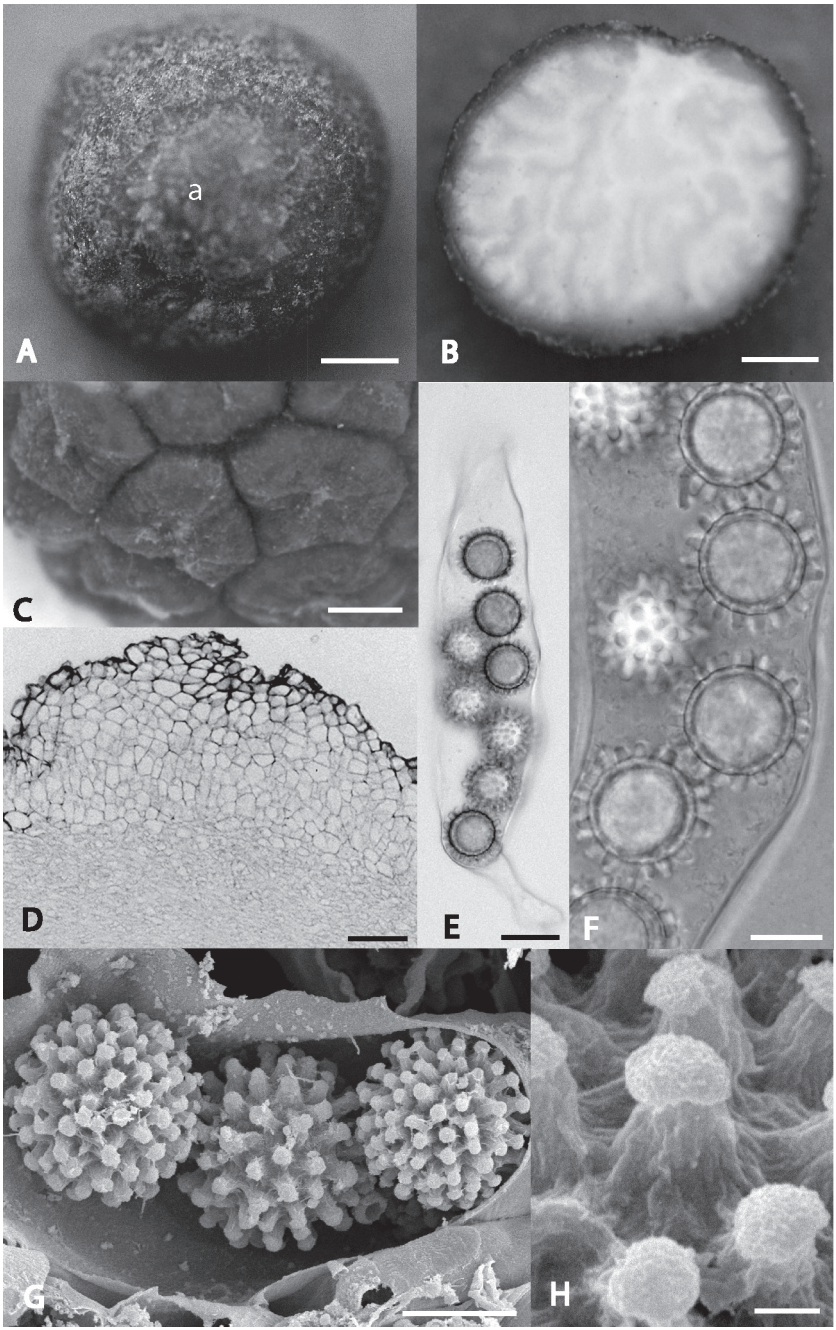
Taxonomy

Pachyphloeus marroninus Healy, Bonito & G. Guevara, **sp. nov.**

MycoBank MB 511872 GenBank EU427549 Figs. 1 A–H

Ptychothecium subglobosum, usque ad 1.7 × 1.5 × 1.3 cm, ordore ingrato, pagina verrucosa, marronina. Excipulum ectale textura angulari; excipulum entale textura intricati. Gleba solida, alba, venis fertilibus, in siccitate cremicolor. Asci clavati, haud amyloidei, octospori, in vallo dispositae. Sporae hyalinae, globosae, 19–22 µm latae ornamentum spinarum includens (1.5–)2–2.5(–3) µm altarum.

Fig. 1. *Pachyphloeus marroninus* images. A. Ascocarp as viewed from top, showing areole (a). (Scale bar = 3 mm) B. Ascocarp longisection showing gleba with opaque white sterile veins and translucent pallid fertile veins. (Scale bar = 3 mm) C.–F. Light microscopy. C. Pyramidal wart-like warts on the peridium. (Scale bar = 0.5 mm) D. Longisection of ascocarp showing an excipular wart composed of textura angularis. (Scale bar = 100 µm) E. Ascus. (Scale bar = 20 µm) F. Ascus with details of spores. (Scale bar = 10 µm) G–H. Scanning electron micrographs of spores and spore spines. G. Spores in an ascus. (Scale bar = 10 µm) H. Single spine of a spore. (Scale bar = 1 µm)


a
A
B
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Holotypus: USA, Iowa, Winneshiek Co., Upper Iowa River Public Access Area 2 October1998, *RH286* (ISC); Isotype (OSC)

Etymology: Latin, *marroninus* (maroon) refers to the maroon color of the ascocarp.

Technical description of holotype

Macrocharacters—Ptychothecium (ascomata) radially symmetrical to slightly compressed, 1.7 × 1.5 × 1.3 cm broad (Fig. 1A, B), sculptured with low, flat, polygonal warts with 4–5 sides, each about 0.3–1 mm broad (Fig. 1C), "Morocco red" (moderate reddish-brown) to "maroon" (dark reddish-brown); ascomal apex with a relatively smooth, "orange-rufous" (strong orange) areole (Fig. 1A a) 11 × 9 mm where internal sterile veins emerge, and a white to cream-colored basal mycelial tuft; odor none at first, of burned potatoes when mature, or when enclosed in a container for several hours. Gleba white with translucent whitish veins at all stages (Fig. 1B), ascus-lined veins becoming "light drab" to cream-colored with drying.

Microcharacters—Ectal excipulum (peridium) reddish-brown as viewed under bright field light microscopy, 368–630 µm thick excluding warts, warts 158–263 µm high, composed of textura angularis (pseudoparenchyma) with cells ± isodiametric, 26–35 µm broad, the walls of the outermost cell 2.5–4 µm thick, thinning on interior cells, grading to the ental excipulum (subhymenium) of hyaline textura intricata next to gleba with hyphae 7–10 µm broad (Fig. 1D). Basal tuft of mycelia 5–10 µm broad, some with granular incrustations. Glebal hyphae 7–10 µm at the septa, some cells swollen to 12 µm. Asci forming a palisade, clavate to clavate-cylindrical, usually slightly ventricose, pedicellate; 162.5–210 × 35–40 µm, with 8 irregularly uniseriate to biseriate spores (Fig. 1E), non-reactive to Melzer's solution with pretreatment in 3% KOH. Spores globose (Figs. 1E-H), 14–17 µm (avg. 15.5 µm), mostly 16–17 µm excluding spines, 19–22 µm (avg. 20.3 µm) mostly 20 µm including spines; spore wall 1.5–2 µm thick, hyaline to chlorinous, and few greenish yellow, ornamented with coarse, acute to capitate spines (1.5–)2–2.5(–3) µm tall, 1–2 µm wide at spine base (Fig. 1H), occasionally spines joined in 2's; 7–9 spines traverse spore surfaces.

Additional *P. marroninus* collections examined—PARATYPES: UNITED STATES. Iowa: Boone Co., Ledges State Park, 21 Jul 1999, R.Healy *482* (ISC, OSC). UNITED STATES. Iowa: Winneshiek Co., Cardinal Marsh Wildlife Access Area 23 Oct 1998, R.Healy *299* (ISC, OSC). Similar collections examined: MEXICO. Nuevo Leon: Municipio de Santiago, El Cercado 14 Sep 1983 J. García *3757* (UNL), Col. Los Pescadores, 10 Aug 1983 J.García *3764* (ITCV). Tamaulipas: Municipio de Ciudad Victoria, Torre de Microondas "Las Mulas", 11 Nov 2006 G. Guevara *891,* G.Guevara *896* (ITCV) Tlaxcala: 1 km E of San Francisco Temezontla, Municipio Panotla Tlaxcala (elevation 2600 m, lat 19 20 76, long 98 16 42), 20 Sep 2007 E. Cazares, *JT32454* (TLXM).

Comments: The paratypes from Iowa were similar to the holotype in all respects. Macroscopically, the Mexican collections were similar to *P. marroninus* except the ascoma were deeper gray-brown in color, lacking the reddish hues of the Iowa collections. Spore spines of Garcia3757 average 1.5 µm tall on average. Spore spines of JT32454 were only 1 µm tall on average.

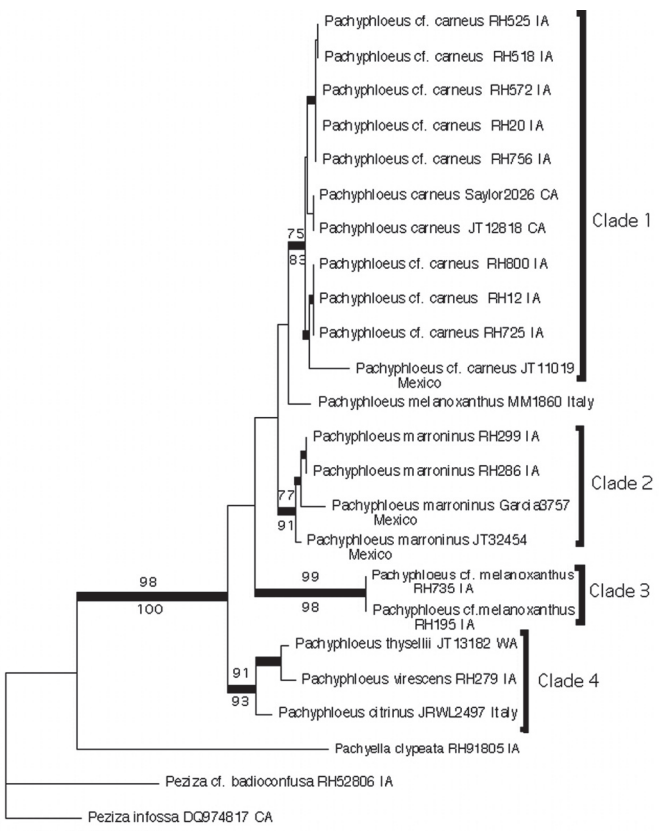

Fig. 2. Phylogenetic reconstruction of the genus *Pachyphloeus* based on ITS and LSU rDNA from 22 taxa, showing the placement of *Pachyphloeus marroninus*. Topologies based on parsimony and maximum likelihood were congruent at all supported nodes; the most likely tree is presented here. Thickened branches indicate those nodes that are supported by bootstrap values of 70% or more. Numbers on the nodes are maximum parsimony (top) and maximum likelihood (bottom) bootstrap values. Taxa are labeled by their collection name and location.

Discussion

Clade 1 is composed of *Pachyphloeus* with orange-colored ascomata with flattened polygonal warts and brown spores. The only *Pachyphloeus* described with an orange ascoma is *Pachyphloeus carneus* from California (Harkness 1899). Helen Gilkey sent a specimen of *P. carneus* to Eduard Fischer, who compared it with the type of *P. citrinus* and determined them to be synonymous (Gilkey 1916). Gilkey tentatively accepted his opinion but with reservations, because the ascomata of North American specimens were consistently bright orange in color compared to the dark brown ascomata with yellow peridial papillae described for European specimens (Berkeley & Broome 1846). She also noted North American specimens were larger. In addition, *P. citrinus* has rounded to cone-shaped warts on the ascoma, rather than the polygonal ones of the species in clade 1. Subsequently, orange *Pachyphloeus* species, such as those included in our study, have been identified as *Pachyphloeus citrinus*. Our results indicate the species in clade 1 are distinct from a *P. citrinus* from Italy in clade 4. We submit that the name *P. citrinus* has been misapplied to the orange-colored species of *Pachyphloeus* from North America and Mexico, and that *P. carneus* is the correct name for this species. Diversity is apparent in this clade, and subsequent morphological and molecular analysis of additional collections may reveal more species.

Clade 2 is represented by *Pachyphloeus marroninus*. The holo- and paratypes of *P. marroninus* differ from other species of *Pachyphloeus* in the combination of dark reddish-brown peridium with polygonal warts, orange areolar area, white gleba with pallid veins at maturity, narrowly clavate asci, and hyaline to chlorinous spores with coarse spines that do not tend to form obviously inflated tips or perisporium. Sequences differ slightly between the Mexican and Iowa collections, which prompted us to look more carefully for morphological differences. Macroscopically these collections are similar in size, and peridial structure. The Iowa collections have a reddish-orange hue, whereas the Mexican collections are dark grayish brown. The spore spines also differ in length. This may be a cryptic species complex that needs analyses of additional collections.

Details of the spines for most *Pachyphloeus* spp. are difficult to distinguish with light microscopy because of the small spore size (averaging 17–20 µm) and complex ornamentation. Adding to the difficulty in viewing spore details, some species have a perisporial covering that develops along the inside surface of the outer delimiting membrane, as is the case in *P. carneus* (Healy 2002). This perisporium obscures the shape of the spines, and makes the interpretation of them when viewed with light microscopy confusing. *Pachyphloeus marroninus* lacks this perisporium; the coarseness of its spines and absence of a perisporium render its spore ornamentation distinct.

Among described species that have similarly colored ascomata, *Pachyphloeus lateritius* differs in having minutely warted spores; *P. austro-oregonensis* has yellow veins, and canal-like openings in the gleba; *P. thysellii*, has a minutely verrucose ascoma, yellow veins and patches among the excipular warts, and spores with very fine spines. All other species are easily differentiated by the colors of both ascoma and gleba.

Clade 3 is composed of only two collections of a single species from Iowa, similar in color to *P. melanoxanthus*, but not clustering with that species in the phylogram. This species is as yet undescribed.

Clade 4 is composed of three species from Washington, Iowa and Italy. Morphological features shared by *P. thysellii* and *P. virescens* include brown ascomata with conical warts, yellow sterile veins and subglobose asci. *P. citrinus* has most of these features, but its asci are clavate.

In general, only those who seek truffles find them. Consequently, collection sites of a species are often far apart. Oaks are the most frequently cited associates of *Pachyphloeus* spp. (Fogel & States 2002, Montecchi & Sarasini 2000), and are the predominant canopy species for collections in Iowa and Mexico. Frank et al. (2006) demonstrated the mycorrhizal relationship of *P. austro-oregonensis* with *Quercus garryana* and reported DNA of this species in scats of mycophagous deer mice (*Peromyscus maniculatus*), a common species in woodlands and prairies from Canada to Central Mexico. The main dispersal mode of truffles is via mycophagy by small woodland mammals (Trappe & Claridge 2005). We hypothesize that *P. marroninus* occurs in oak woods from Iowa into Mexico, but data are missing from woodlands between the collection sites for lack of searching. Oaks presently occur contiguously from Canada to the Rio Grande River and into Mexico. Even disjunct oak communities in Mexico are remnants of more extensive forests and woodlands in the Tertiary and Quaternary (Valiente-Banuet et al. 2006). Hence the present disjunct distribution of *P. marroninus* probably reflects a historically continuous distribution. Moreover, transport of spore-bearing small mammals by preying raptors could account for long distance dispersal between separated oak stands (Trappe & Claridge 2005).

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